REPORT DOCUMENTATION PAGE

Form Approved OMB NO. 0704-0188

The public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggesstions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA, 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any oenalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.
PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.

1. REPORT DATE (DD-MM-YYYY) 03-05-2010	2. REPORT TYPE Final Report	3. DATES COVERED (From - To) 1-Jul-2006 - 31-Dec-2009

4. TITLE AND SUBTITLE
Biomolecular Mechanism, Cloning, Sequencing and Analysis of Adaptive reflection cDNAs and Proteins from Squid

5a. CONTRACT NUMBER
W911NF-06-1-0285

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER
611102

6. AUTHORS
Daniel E. Morse

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAMES AND ADDRESSES
University of California - Santa Barbara
Office of Research
University of California
Santa Barbara, CA   93106 -2050

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Research Office
P.O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSOR/MONITOR'S ACRONYM(S)
ARO

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
49623-LS.4

12. DISTRIBUTION AVAILIBILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
The views, opinions and/or findings contained in this report are those of the author(s) and should not contrued as an official Department of the Army position, policy or decision, unless so designated by other documentation.

14. ABSTRACT
We report our investigations elucidating the molecular mechanisms underlying the adaptive reflectin-based biophotonic system in squids
governing the dynamic changes in reflectance and structural color of these animals. We discovered that a neurotransmitter-activated signal
transduction cascade culminating in phosphorylation of the reflectin proteins (that we cloned and sequenced) drives

15. SUBJECT TERMS
Biomolecular Mechanism, Adaptive Reflectance, Adaptive Optics, Reflectin, Bragg Reflectors, cDNA, Protien, Squid, Cephalopod, Octopus

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	15. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON Daniel Morse
a. REPORT UU	b. ABSTRACT UU	c. THIS PAGE UU	UU		19b. TELEPHONE NUMBER 805-893-3157

Standard Form 298 (Rev 8/98)
Prescribed by ANSI Std. Z39.18

<div align="center">**Report Title**</div>

Biomolecular Mechanism, Cloning, Sequencing and Analysis of
Adaptive reflection cDNAs and Proteins from Squid

<div align="center">**ABSTRACT**</div>

We report our investigations elucidating the molecular mechanisms underlying the adaptive reflectin-based biophotonic system in squids governing the dynamic changes in reflectance and structural color of these animals. We discovered that a neurotransmitter-activated signal transduction cascade culminating in phosphorylation of the reflectin proteins (that we cloned and sequenced) drives the condensation of these proteins, with consequent changes in the dimensions of the membrane-enclosed lamellae that form the reflective Bragg reflectors in the iridophore cells in the skin, thereby activating reflectance and dynamically tuning its color. We also discovered a related reflectin-mediated biophotonic system producing omni-directional and multi-wavelength mirror-like reflectance. Results of this project have revealed new mechanisms by which biomolecular systems dynamically control light, and these mechanisms in turn are enabling the design and synthesis of a new generation of electro-optical materials and devices.

List of papers submitted or published that acknowledge ARO support during this reporting period. List the papers, including journal references, in the following categories:

<div align="center">**(a) Papers published in peer-reviewed journals (N/A for none)**</div>

Izumi, M., A. M. Sweeney, D. G. DeMartini, J. C. Weaver, M. L. Powers, A.R. Tao, T. V. Silvas, R. M. Kramer, W. J. Crookes-Goodson, L. M. Mäthger, R. R. Naik, R. T. Hanlon and D. E. Morse (2010). Changes in reflectin protein phosphorylation are associated with dynamic iridescence in squid. Interface - J. Royal Soc. 7: 549-560.

Tao, A.R., D. G. DeMartini, M. Izumi, A. M. Sweeney, and D. E. Morse (2010). The role of protein assembly in dynamically tunable bio-optical tissues. Biomaterials (in press).

Number of Papers published in peer-reviewed journals: 2.00

<div align="center">**(b) Papers published in non-peer-reviewed journals or in conference proceedings (N/A for none)**</div>

Number of Papers published in non peer-reviewed journals: 0.00

<div align="center">**(c) Presentations**</div>

D.E. Morse:

11/06   Invited Lecture, "Biotechnology Opens New Routes to High-Performance Materials for Improved Photovoltaics, Batteries, Uncooled IR Detectors, Ferroelectrics and Optical Applications"
25th Army Science Conference, Orlando, FL

05/07   Invited Talk, "Bio-Inspired Engineering: from Semiconductor Nanofabrication to Dynamically Adaptive Bio-Photonics"
"BioExploitation: Better Engineering Through Biology" DARPA Workshop, Arlington, VA

09/07   Invited Talk, "Bio-Inspired Engineering: from Semiconductor Nanofabrication to Dynamically Adaptive Bio-Photonics"
2nd International Workshop on Approaches to Single-Cell Analysis, Tokyo, Japan

09/07   Invited Speaker, "Hierarchical Self-Assembly, Catalytic Nanofabrication, Adaptive Biophotonics" Biomolecular Science & Engineering Program Retreat, UCSB

01/08   Invited Talk, "Bio-Inspired Materials Engineering: From Semiconductor Nanofabrication to Dynamically Adaptive Bio-Photonics"
Heraeus Winter School Functional Hybrid Materials Design Bremen, Germany

02/08   Invited Lecture, "Biologically Inspired Low-Temperature Nanofabrication of Semiconductor, Ferroelectric and Perovskite Thin Films and Nanoparticles; Adaptive Optical Materials"   MROP 2008, UCSB

06/08   Invited Lecture, "Bio-Inspired Nanofabrication of Semiconductors and Dynamically Adaptive Optical Materials"   3rd International Conference "Smart Materials, Structures and Systems", Acireale, Catania Italy

08/08   Invited Lecture, "Dynamically Adaptive Biophotonics: Protein Phosphorylation Drives Changes in Iridescence in Squid"
     American Chemical Society Symposium, Philadelphia, PA

11/08   Invited Lecture, "Dynamically Adaptive Biophotonics: Protein Phosphorylation Drives Changes in Iridescence in Squid Inspiring New Approaches to Dynamically Tunable Optical Materials"   BioNano Workshop, Kawagoe, Japan

11/08   Invited Lecture, "New Pathway for Encoding Self-Assembly and Emergent Properties"   Molecular Foundry User Meeting 2008, Berkeley, CA

12/08   Invited Lecture, "Dynamically Adaptive Biophotonics: Protein Phosphorylation Drives Changes in Iridescence in Squid, Inspiring New Approaches to Dynamically Tunable Optical Materials"   3rd BioNano Workshop, Kawagoe, Japan

01/09   Invited Talk, "New Pathways for Encoding Self-Assembly and Emergent Properties", Daniel E. Morse, Igor Mezic, Meredith Murr, Gunjan Thakur   Society for Biological Engineering 2nd International Conference on Biomolecular Engineering, Santa Barbara, CA

02/09   Invited Lecture, "Dynamically Adaptive Biophotonics: Protein Phosphorylation Drives Changes in Iridescence in Squid, Inspiring New Approaches to Dynamically Tunable Optical Materials; and New Pathways for Encoding Self-Assembly and Emergent Properties"
     Knight Lecture University of Akron, Akron OH (Advanced Lecture)

04/09   Invited Speaker, "Dynamically Tunable Biophotonics: Camouflage and Communication in Squid Inspire New Approaches to Tunable Optical Materials"   MCDB Seminar, UCSB

10/09   Invited Plenary Lecture, "Nanotechnology in Nature"   INSPIRE BioNano International Conference, Dublin, Ireland

10/09   Invited Lecture, "Biological Systems Inspire New Pathways to High-Performance Materials for Energy and Photonics"
Bioengineering Insights 2009, UCSB

Supervised by D. Morse:

01/07   Invited Poster, "Active Biophotonics: Reflectin Phosphorylation Drives Adaptive Reflectance in Squid" Michi Izumi, James C. Weaver, Meghan L. Powers, Tania V. Silvas, Woo R. Suh, Daniel E. Morse, presented by Michi Izumi   Materials Research Outreach Program Symposium, UCSB

08/07   Invited Poster, "Phosphorlyation of Reflectin Proteins Associated with Changes in Adaptive Reflectance in the Squid, Loligo pealeii", Michi Izumi, James C. Weaver, Daniel E. Morse, presented by Michi Izimi   234th ACS National Meeting, Boston, MA

02/08   Invited Poster "Bio-Inspired Materials for Lightweight Portable Energy and Other Army Applications", Birgit Schwenzer, Andrea Tao, Richard Brutchey, Qian Gu, James R. Neilson, and Daniel E. Morse, presented by Birgit Schwenzer   2008 ICB/Army/Industry Collaboration Conference, UCSB

04/08   Invited Talk, "Biophotonics: The Adaptive Response of Squid Skin", Andrea R. Tao, Michi Izumi, Daniel DeMartini, and Daniel E. Morse, presented by Andrea R. Tao   UCOP Fellowship Retreat, Lake Arrowhead, CA

07/08   Invited Talk, "Plasmonic Lattices", Andrea Tao   Gordon Research Conference on Plasmonics, Tilton, NH

01/09   Invited Talk, "Patterns of S-crystallin Evolution are Correlated with Optical Acuity in Cephalopods", Alison Sweeney, Mikhail V. Matz, Daniel E. Morse, S. Johnsen, presented by Alison Sweeney   Society for Integrative and Comparative Biology Annual Meeting, Boston, MA Presented in affiliation with special "Biomaterials" Symposium sponsored by SICB

04/09   Invited Talk, "Self-Assembled Plasmonic Crystals", Andrea R. Tao, Daniel P. Ceperley, Andrew M. Neureuther, Peidon Yang, presented by Andrea R. Tao   6th Annual Foundations of Nanoscience Conference on Self-Assembled Architecture and Devices, Snowbird, UT

05/09   Invited Talk, "Biological Inspiration for Dynamically Adaptive Camouflage", Alison Sweeney, Daniel DeMartini, Michi Izumi, Amanda Holt, and Daniel E. Morse, presented by Alison Sweeney   Reliance 21 Materials & Processes, Research and Engineering,

Technology Focus Team, Annual Planning Workshop, St. Michaels, MD

05/09   Invited Lecture, "Lens Design and Iridescence: Evolution and Biophysics of Soft Photonic Structures in Invertebrates" presented by Alison Sweeney   EEMB Seminar, UCSB

08/09   Invited Poster, "Adaptive Bio-Optics: The Role of Dynamic Protein Assembly in Squid Skin Coloration", Andrea R. Tao, Daniel G. DeMartini, and Daniel E. Morse, presented by Andrea R. Tao   42nd IUPAC Congress, Glasgow, Scotland

09/09   Invited Poster, "Adaptive Bio-Optics: The Role of Dynamic Protein Assembly in Cephalopod Coloration, Andrea R. Tao, Daniel G. DeMartini, and Daniel E. Morse, presented by Daniel G. DeMartini   BMSE Fall Retreat, UCSB

10/09   Invited Poster, " Changes in Reflectin Phosphorylation Drives Iridescence in Squid", Daniel DeMartini, Michi Izumi, Andrea Tao and Dan Morse, presented by Daniel DeMartini   BioEngineering Insights Conference, Santa Barbara, CA

01/10   Invited Talk, " Deep-Sea Silver: Photonics and Biochemistry of Semi-Coherent Broadband Reflectors on Squid Eyes", Sweeney, A.M., Holt, A.L., Mason, E., and Morse, D.E., presented by Alison M. Sweeney   Society for Integrative and Comparative Biology Symposium, Seattle, WA

Number of Presentations: 29.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):   0

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):   0

(d) Manuscripts

Holt, A.L., J. G. A. Wehner, A. Hammp and D. E. Morse. 2010. Plastic transmissive infrared electrochromic devices. Macromolecular Chemistry & Physics. (submitted)

Number of Manuscripts: 1.00

Number of Inventions:

Graduate Students

NAME	PERCENT SUPPORTED
Daniel DeMartini	0.80
FTE Equivalent:	**0.80**
Total Number:	**1**

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
Ralph Imondi	0.05
Amanda Holt-Jones	0.05
FTE Equivalent:	**0.10**
Total Number:	**2**

Names of Faculty Supported

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Under Graduate students supported

NAME	PERCENT SUPPORTED	
M. L. Powers	0.20	
T.V. Silvas	0.20	
Woo R. Suh	0.20	
FTE Equivalent:	**0.60**	
Total Number:	**3**	

Student Metrics
This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period: 3.00

The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields: 3.00

The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields: 3.00

Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale): 3.00

Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering: 0.00

The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense 0.00

The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields: 3.00

Names of Personnel receiving masters degrees

NAME
Total Number:

Names of personnel receiving PHDs

NAME
Total Number:

Names of other research staff

NAME	PERCENT SUPPORTED	
Anna Howell	0.05	No
FTE Equivalent:	**0.05**	
Total Number:	**1**	

Sub Contractors (DD882)

Inventions (DD882)

FINAL REPORT
Biomolecular Mechanism, Cloning, Sequence & Analysis
of Adaptive Reflectin cDNAs & Proteins from Squid

Grant # W911NF-06-1-0285

Daniel E. Morse

TABLE OF CONTENTS

A.1. Scientific Objectives.

Our overall objective is to discover and harness the previously unknown molecular mechanisms that govern the remarkable capabilities for intraspecies communication in squid as a model for new materials and modalities for optoelectronic communication. The immediate research goal embodied in this proposal is to identify the biomolecular mechanisms responsible for neuronally activated changes in reflectin protein conformation, association, modification and/or cofactor binding leading to rapidly controllable changes in color or reflectance. Candidate drivers of these adaptive changes in reflectance that we will investigate include electromotive ion fluxes, pH change and exposures to metal ions and biological signal- and energy-transduction molecules.

Our long-term goal, toward which this proposal represents the first step, is to discover the underlying molecular basis for the remarkably rapid adaptive changes in reflectance exhibited by this system, and to then (in collaborative research with materials scientists and engineers that is beyond the scope of this present proposal) design and synthesize materials that translate this mechanism into practical engineering, chemistry and physics. (Such translation of biomolecular mechanisms underlying the synthesis and performance of biomolecular materials has been the theme and motivation for my research for the past 12 years, first with the abalone shell, and then with the mechanism of biosilica synthesis, translating these to new routes for semiconductor synthesis; cf. examples below.) If, for example, we were able to confirm the hypotheses that reversible metal binding, modification or reversible swelling of specific proteins (as just three hypotheses) might be involved in the reversible changes in reflectance, this would guide our efforts to develop a material that could do something similar in response to changes in applied electrical potential.

A.2. Results with Highest Importance and Relevance to Army Needs.

Cephalopods (such as octopi and squid) exhibit a remarkable ability to rapidly change their appearance for communication or camouflage. Neural modulation of the skin alters the pigmented chromatophore patterns and their complementary iridescence, which emanates from lamellar Bragg-like thin-film reflectors in cells called iridophores. Changeable iridescence is achieved via a muscarinic cholinergic system in which the neurotransmitter acetylcholine (ACh) and calcium ion induce changes in the "reflectin" protein-based reflecting platelets of the iridophores. In the first year of this project we have cloned and sequenced the cDNAs coding for two reflectin proteins from a squid that exhibits dynamically adaptive changes in reflectance, and identified unique features of these proteins that are distinct from the reflectins present in a species that does not exhibit dynamically changeable reflectance. We discovered that the reflectins are highly phosphorylated, and that this phosphorylation increases markedly in response to ACh stimulation, concomitant with the induced increase in reflectance. We found that genistein, a protein tyrosine kinase inhibitor, blocks the Ach- and calcium-induced increase in phosphorylation of the reflectins, and the resulting iridescence. Our results suggest that phosphorylation of tyrosine residues of one or more proteins participates as a signal transducer, acting as a molecular switch governing the ACh- and calcium-induced dynamic change in iridescence in the squid *Loligo pealeii*. Our results indicate that binding of ACh elicits activation of G-protein mediated production of inisotol 1,4,5-trisphosphate (IP_3) by activation of phospholipase C (PLC). IP_3 then elevates the amount of calcium that can bind to calmodulin to activate protein tyrosine kinases (PTK).   These kinases ultimately phosphorylate reflectin

proteins, leading to changes in conformation and hierarchical assembly within the disc-like Bragg reflectors in the iridophores that produce the ultimate changes in reflectance. We also succeeded in modeling the kinetics of the rise and fall in calcium concentration that mediates the neuronally activitated phosphorylation of reflectin proteins and the consequent increase in reflectance in the skin of cephalopods.

These results will help in the design of biologically inspired synthetic optoelectronic materials and devices for adaptive optical switching in applications important to Army operations. A new partnership with Raytheon is exploring the application of these findings for practical device development; this collaboration already has resulted in one publication (Holt et al., 2010, op. cit.) and a seedling grant from ARL.

A.3. **Brief Scientific Rationale**.
Iridophore cells of certain cephalopods respond to the neurotransmitter, acetylcholine (ACh), by changing their reflectance for signaling via an optical communication pathway, and for camouflaging to match their optical background (1). In this funding period we succeeded in experimentally elucidating the signal transduction pathway that controls this response; cloning and sequencing the genes coding for the proteins that reversibly form the tunable Bragg reflectors responsible for the dynamically adaptive optical changes in reflectance; and mathematically modeling this control. We anticipate that translation of these results to synthetic systems will enable the design of biologically inspired, dynamically adaptive optoelectronic materials and devices for optical switching in applications important to Army operations.

A.4. **Detailed Research Methods and Results.**
Iridescence in the skin of the squid, *L. pealeii,i* can be induced by the exogenous addition of the neurotransmitter, ACh (Fig. 1A, left). Rapid changes in both the intensity and color of reflectance are observed following the addition of ACh. As reported previously for *L. brevis,* we found that the calcium ionophore A23187 also can induce iridescence in *L. pealeii* without the addition of ACh (Fig. 1, upper), suggesting that intracellular calcium plays an important role in the activation of adaptive iridescence in squid. The spectral shift induced by the addition of ACh was observed to proceed from reddish (680 nm) to orange-yellow (650 nm) (Fig. 2A right). This is a unique form of adaptive iridescence described only in squid.

Because the ACh-induced increase in iridescence in a related species of squid had been shown to proceed by activation of a muscarinic ACh receptor and appeared to involve calcium ion as a signal transducing second messenger and because such pathways typically depend upon the activation of specific protein kinases (21), we tested the sensitivity of this induction to several inhibitors of protein tyrosine kinases (PTKs). The inhibitors we tested included blockers of the Src and MAP kinases, and of protein kinase C (PKC), a Ser/Thr kinase known to be activated by both diacylglycerol (DAG) and calcium ions. Although specific inhibitors for PKC, Src and MAP kinase did not affect the ACh-induced iridescence (data not shown), genistein, a broad-range tyrosine kinase inhibitor (22), dramatically suppressed the ACh-induction of iridescence (Fig. 2A). This effect of genistein exhibited a clear dose dependency (Fig. 2B).

Because of this apparent dependence of the ACh-induced iridescence on protein phosphorylation, we sought to characterize potential targets in the iridophore layer of the skin. Although refractive index measurements had suggested to Denton and Land that the iridosomes

in squid are composed of chitin (5), subsequent evidence revealed that they are proteinaceous (15, 16, 18). Our analyses of the insoluble material extracted from the iridophore-containing layer of skin from *L. pealeii* could detect no evidence for chitin; no glucosmine (the signature product of hydrolysis of chitin) was found after complete acid hydrolysis (data not shown). We were, however, able to extract and characterize four insoluble proteins that are unique to the iridophore-containing layer of the skin (Fig. 3A, left). Two of these proteins, with apparent molecular masses of 40 kDa and 25 kDa based on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) mobility, cross-react with anti-reflectin antibodies that had been generated against *E. scolopes* reflectins (Fig. 3A, light). Genes coding for these two proteins were cloned (23) and their deduced amino acid sequences were analyzed (Fig. 3B, revealing that they exhibited high similarity to the reflectin proteins of *E. scolopes* and *Loligo forbesi* (24). Based on these observations, these two genes were designated as Ref-Lp1 and Ref-Lp2 (for Reflectin-*Loligo pealeii* 1 and 2, respectively). Although seven distinct reflectin gene variants were identified from *E. scolopes*, a similar diversity of reflectins has not yet been found in *L. pealeii*.

As is the case for the other reflectin proteins (16), Ref-Lp1 and 2 contain a series of conserved subdomains (SDs). Ref-Lp1 is composed of six SDs [M/FD(X)$_5$MD(X)$_5$MDX$_{3/4}$] and Ref-Lp2 is composed of four SDs (Fig. 2). Hydropathy plots of Ref-Lp1 and 2 revealed that these two proteins are globally hydrophilic (data not shown), lacking any distinct hydrophobic regions, demonstrating that they contain none of the specific characteristics of trans-membrane proteins. Our analyis of these sequences using software that predicts the existence of membrane-associated protein domains also proved negative, supporting this observation. The Ref-Lps are highly insoluble in aqueous systems, however, and are fractionated as insoluble proteins with the cellular membrane fraction. The calculated isoelectric points (pI) for the two Ref-Lp proteins are >9, indicating that these proteins are likely to be positively charged under physiological conditions.

In addition to the two reflectins, we discovered two other unique iridophore-layer-specific proteins, designated IN-1 and IN-2 (ImmunoNegative proteins 1 and 2), because they do not cross-react with the anti-reflectin antibodies. Preliminary sequence data indicate that these are not members of the reflectin family (16), evidence that is consistent with the negative results obtained from the immunodetection studies. (Sequence analyses of IN-1 and IN-2 will be presented elsewhere.) IN-1 is the most abundant iridophore-layer-specific protein that we have been able to extract from this tissue (Fig. 3A, left).

We discovered numerous potential sites of phosphorylation by analysis of the Ref-Lp1 and Ref-Lp2 sequences with NetPhos2.0, a program used for the prediction of potential protein phosphorylation sites (25). Using this technique, we found 10 serine (Ser) and 13 tyrosine (Tyr) as likely sites of phosphorylation on Ref-Lp1, and 5 Ser, 11 Tyr and 1 threonine (Thr) as possible sites for phosphorylation on Ref-Lp2. In contrat, our analysis of the Ref-Lp1 and Ref-Lp2 sequences using PROSITE (26) revealed no calcium ion binding sites (such as EF-hand motifs) within the reflectin molecules, suggesting instead that calcium ion may act in this system as a second messenger (cf. Fig. 1), rather than by interacting directly with the reflectins.

Because NetPhos2.0 is designed based on the entire eukaryotic kinase database, and no group-specific database for cephalopods has yet been established, we expected that NetPhos2.0 might

indicate some false positives. However, phosphorylation of both Ref-Lp1 and Ref-Lp2 was confirmed and quantified directly with Pro-Q Diamond, a stain specific for phosphoamino acids; these analyses were performed following separation of the proteins by SDS-PAGE. Comparing the signal intensities between samples prepared from activated (iridescent) and non-activated (non-iridescent) iridophore layers, we found that both Ref-Lp1 and Ref-Lp2 showed higher phosphorylation levels in their activated state (Fig. 4A, left). Since Pro-Q Diamond cannot differentiate between different phosphorylated amino acids, we further analyzed the proteins by reaction with a phosphotyrosine-specific antibody, PY20 (Fig. 4A, right). Results from these studies revealed that tyrosine residues on both Ref-Lp1 and Ref-Lp2 are phosphorylated, and that the phosphorylation level of tyrosine residues in activated Ref-Lp2 was 1.8-fold greater than in the non-activated form. Ref-Lp1 exhibited a lesser but still detectable increase in PY20 staining intensity. To specifically identify the locations and identities of some of the phosphorylated residues, mass spectroscopic (MS) mapping was performed following trypsin digestion of Ref-Lp1 and Ref-Lp2 using matrix-assisted laser desorption/ionization technique time-of-flight mass spectrometry (MALDI-TOF MS). MS mapping revealed that Tyr14 and Tyr127 on Ref-Lp1 and Tyr12, Tyr214, Ser218 and Tyr223 on Ref-Lp2 were phosphorylated in the activated state (Fig. 4B). All of these phosphorylated residues were predicted as possible phosphorylation sites by NetPhos 2.0; interestingly, they all occur outside the conserved (SD) regions of the reflectins. It is possible that additional phosphoamino acids may have been missed by our analyses due to lability during protein isolation and purification (27, 28).

Further biochemical and immunochemical analyses confirmed the high degree of phosphorylation of both reflectin proteins from *L. pealeii,* suggesting that at least some of this phosphorylation may be related to the dynamically tunable ACh-controlled iridescence. Since more than one phosphorylated residue was identified, two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) was performed to determine if these residues might exhibit differential phosphorylation before and after activation with ACh. These analyses (Fig. 5A) revealed that Ref-Lp1 and Ref-Lp2 each consist of populations with several distinct phosphorylated states. _Immunodetection with PY20 revealed that the most acidic Ref-Lp2 molecules, corresponding to the most phoshorylated molecules (barely detected by Pro-Q Diamond staining due to their limited quantity), were in significantly higher abundance in the activated state (Fig. 5B), while the most basic and least phosphorylated molecular species was the dominant form revealed by this stain. Interestingly, IN-1 also is phosphorylated and dephosphorylated, but *reciprocally* with the ACh-dependent phosphorylation of the reflectins (Fig. 4A, left and 5A). Preliminary analyses of phosphorylation on IN-1 revealed that unlike the reflectins, IN-1 contains very few phosphorylated tyrosine residues (22), suggesting that other amino acids (most likely Ser and Thr) are the primary sites of phosphorylation in this protein. Partial direct sequence analysis of IN-1 reveals that this protein exhibits no apparent sequence homology to any known proteins, suggesting that it may be unique to the squid's adaptive iridescence system. Contrary to the results obtained from IN-1, IN-2, which also fails to cross-react with anti-reflectin antibodies, appeared to be increasingly phosphorylated following addition of ACh (Fig. 5A). Ref-Lp1 was difficult to detect reproducibly in 2-D PAGE, because this protein thus far can only be solubilized in solutions containing the detergent SDS, which is incompatible with IEF gel chemistry.

4

Concomitantly with its inhibition of adaptive reflectance (cf. Fig. 2), genistein also reduced the phosphorylation of Ref-Lp2 (Fig. 4A). Quantification of phosphorylation with Pro-Q Diamond and PY20 revealed that genistein treatment suppressed the ACh-activated phosphorylation of Ref-Lp1 and Ref-Lp2 to levels equal to or lower than in the non-ACh activated samples. Because protein phosphorylation is generally balanced by active dephosphorylation, it is not surprising that genistein inhibition of phosphorylation is seen to reduce the phosphate content below that of the non-activated controls. These results demonstrate that phosphorylation of Ref-Lps is correlated with adaptive iridescence, and that control of phosphorylation - of one or more proteins yet to be identified- could be involved in the switching mechanism (29). Because we found that ACh induces a rapid, marked reduction in the phosphorylation of IN-1 at the same time that Ref-Lp2 is being phosphorylated, it is possible that IN-1 may function as a phosphate donor, driving changes in phosphorylation of the reflectin or other proteins critical for the adaptive increase in reflectance.

Phosphorylation is known to trigger dramatic changes in the conformations and assembly of many proteins (30); such changes could alter the refractive index (31), thickness and spacing (1, 5, 15) of the iridosome that constitute the Bragg-like molecular reflectors in this system. Alternatively, phosphorylation may trigger changes in the Gibbs-Donnan equilibrium across the iridosome membranes, thereby affecting the observed ACh-induced changes in reflectance. It may be relevant in this context to note that Chah *et al.* reported that structural changes of proteins attached to gold nanoparticles in suspension caused changes in refractive index and color in that system (32). It was also shown recently that changes in the refractive index of the interstitial fluid surrounding Bragg-like reflectors of butterfly wing scales dramatically changes their iridescent color (33). In squid skin, ACh-induced phosphorylation may affect the assembly dynamics of the reflectin proteins within the iridosomes, inducing protein condensation to form the coherent plate-like structures consistent with those reported previously from TEM studies (18).

The cephalopod system of rapid adaptive coloration is unique for its speed of change and its diversity of appearances and behavioral functions (34). From the viewpoint of the overall appearance of the skin patterns, the structural coloration from iridophores is highly coordinated with the overlying chromatophores (35). Neural control of the overlying chromatophores is quite well known (36) but neural control of the slower-changing iridophores (i.e. milliseconds in chromatophores, seconds in iridophores) remains poorly understood. The source of ACh driving the changes in reflectance in this layer of skin also is unknown; there are ACh receptors on the cell membranes of the iridophore cells, yet neither neurons nor muscles can be seen in electron micrographs to connect to these cells (9, 15, 16). Nevertheless, there must be extensive cellular communication between these skin layers to produce the coordinated array of pigmentary and structural coloration patterns observed. Identification of the protein kinase controlling iridescence, identification of its proximate targets, and additional studies of this optically dynamic, muscarinic system thus can be expected to unveil further details of neuronal and molecular mechanisms controlling this biophotonic system.

In collaboration with Frank Doyle, we recently succeeded in developing a quantitative model that predicts the transient rise in intracellular calcium induced by ACh in this system.

These results will help in the design of biologically inspired synthetic optoelectronic materials and devices for adaptive optical switching in applications important to Army operations. A new partnership with Raytheon is exploring the application of these findings for practical device development; this collaboration already has resulted in one publication (Holt et al., 2010, op. cit.) and a seedling grant from ARL.

References

1.   Denton, E. J. (1971) *Sci. Am.* **224,** 64-72.
2.   Mäthger, L. M., Land, M. F., Siebeck, U. E. & Marshall, N. J. (2003) *J. Exp. Biol.* **206,** 3607-3613.
3.   Osorio, D. & Ham, A. D. (2002) *J. Exp. Biol.* **205,** 2017-2027.
4.   Vukusic, P. & Sambles, J. R. (2003) *Nature* **424,** 852-855.
5.   Denton, E. J. & Land, M. F. (1971) *Proc. R. Soc. London B Biol. Sci.* **178,** 43-61.
6.   Cloney, R. A. & Brocco, S. L. (1983) *Awer. Zool.* **23,** 581-592.
7.   Huxley, A. F. (1968) *J. Exp. Biol.* **48,** 227-245.
8.   Land, M. F. (1972) *Prog. Biophys. Mol. Biol.* **24,** 75-106.
9.   Mirow, S. (1972) *Z. Zellforsch.* **125,** 176-190.
10.   Ide, H. & Hama, T. (1972) *Biochim. Biophys. Acta* **264,** 65-72.
11.   Rohrlich, S. T. & Rubin, R. W. (1975) *J. Cell Biol.* **66,** 635-645.
12.   Kasukawa, H., Oshima, N. & Fujii, R. (1986) *Comp. Biochem. Physiol. C* **83,** 1-7.
13.   Butman, B. T., Obika, M., Tchen, T. T. & Taylor, J. D. (1979) *J. Exp. Zool.* **208,** 17-34.
14.   Greenberg, N. (2002) *Integr. Comp. Biol.* **42,** 526-540.
15.   Cooper, K. M., Hanlon, R. T. & Budelmann, B. U. (1990) *Cell Tissue Res.* **259,** 15-24.
16.   Crookes, W. J., Ding, L. L., Huang, Q. L., Kimbell, J. R., Horwitz, J. & McFall-Ngai, M. J. (2004) *Science* **303,** 235-238.
17.   Kramer, R. M., Crookes-Goodson, W. J. & Naik, R. R. (2007) *Nature Materials* **6,** 533 - 538.
18.   Hanlon, R. T., Cooper, K. M., Budelmann, B. U. & Pappas, T. C. (1990) *Cell Tissue Res.* **259,** 3-14.
19.   Felder, C. C. (1995) *Faseb J* **9,** 619-25.
20.   Mäthger, L. M., Collins, T. F. & Lima, P. A. (2004) *J. Exp. Biol.* **207,** 1759-69.
21.   van Koppen, C. J. & Kaiser, B. (2003) *Pharmacology & Therapeutics* **98,** 197-220.
22.   Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., Shibuya, M. & Fukami, Y. (1987) *J. Biol. Chem.* **262,** 5592-5595.
23.   **,** Methods used for all DNA cloning, sequence and protein analyses are standard; citations can be found in the supporting online material.
24.   Weiss, J. L., Evans, N. A., Ahmed, T., Wrigley, J. D., Khan, S., Wright, C., Keen, J. N., Holzenburg, A. & Findlay, J. B. (2005) *Biochim. Biophys. Acta* **1668,** 164-174.
25.   Silverman, B. D. (2003) *Protein Sci.* **12,** 586-599.
26.   Gattiker, A., Gasteiger, E. & Bairoch, A. (2002) *Appl. Bioinformatics* **1,** 107-108.
27.   Mikesh, L. M., Ueberheide, B., Chi, A., Coon, J. J., Syka, J. E. P., Shabanowitz, J. & Hunt, D. F. (2006) *Biochimica Et Biophysica Acta-Proteins and Proteomics* **1764,** 1811-1822.
28.   Sickmann, A. & Meyer, H. E. (2001) *Proteomics* **1,** 200-206.

29. The correlation between iridescence and reflectin phosphorylation reported here is supported by the observation that, in the squid *Euprymna scolopes*, which exhibits only passive iridescence, only the reflectin proteins expressed in the reflective light organ (site of colonization by the bioluminescent bacterial endosymbionts) are phosphorylated.

30. Karim, C. B., Zhang, Z., Howard, E. C., Torgersen, K. D. & Thomas, D. D. (2006) *J. Mol. Biol.* **358,** 1032-1040.

31. Zako, T., Harada, K., Mannen, T., Yamaguchi, S., Kitayama, A., Ueda, H. & Nagamune, T. (2001) *J. Biochem. (Tokyo)* **129,** 1-4.

32. Chah, S., Hammond, M. R. & Zare, R. N. (2005) *Chem. Biol.* **12,** 323-328.

33. Potyrailo, R. A., Ghiradella, H., Vertiatchikh, A., Dovidenko, K., Cournoyer, J. R. & Olson, E. (2007) *Nature Photonics* **1,** 123-128.

34. Hanlon, R. T. & Messenger, J. B. (1996) *Cephalopod Behaviour.* (Cambridge University Press, Cambridge).

35. Mäthger, L. M. & Hanlon, R. T. (2006) *Biol. Lett.* **2,** 494-496.

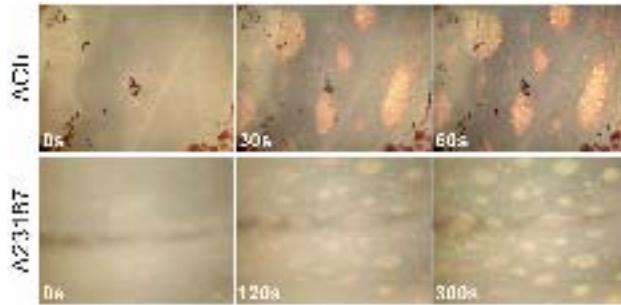36. Messenger, J. B. (2001) *Biol. Rev.* **76,** 473-528.

Figures

Fig. 1. Stimulated iridescence and analyses of platelet components. Iridescence of *L. pealeii* was induced by either ACh (upper) or 15μM A23187 in the presence of additional 4mM CaCl$_2$ (final concentration) in filtered natural seawater (lower). Images were recorded at indicated time points.
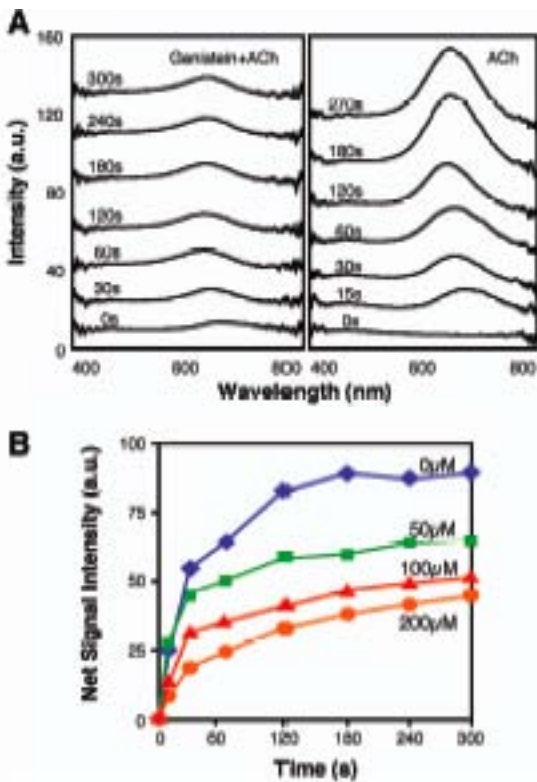

Fig. 2. Effect of genistein (a tyrosine kinase inhibitor) treatment on iridescence.
(*A*) Spectral changes of iridescence for 200μM genistein treated (left) and untreated (right) iridophores following the addition of ACh (10μM). Each spectrum was measured at indicated time point. (*B*) Dose dependency of genistein. Following treatment with genistein at indicated concentrations, iridescence was activated with ACh (10μM).
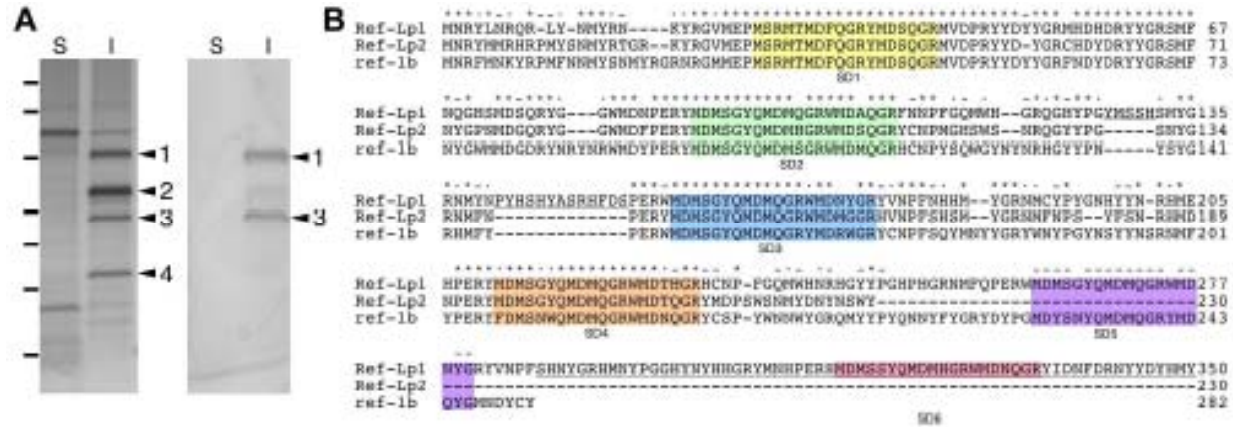
Fig. 3. Purification, cloning and sequencing of the dynamically adaptive reflectin proteins. (*A*) Total iridophore layer-associated proteins were fractionated into soluble and insoluble components and then subjected to SDS-PAGE (left) followed by Western blotting with anti-reflectin antibodies (right). Soluble (S) and insoluble (I) fractions were analyzed separately. The four major iridophore layer-specific protein bands are indicated by numbered arrows. Bands 1 and 3 correspond to Ref-Lp1 and Ref-Lp2 and bands 2 and 4 correspond to the immunonegative bands IN-1 and IN-2, respectively. Bars indicate molecular weight markers of 64, 49, 37, 26, 19 and 15kDa. (*B*) Amino acid sequence alignment of Ref-Lp1 and Ref-Lp2 from *Loligo pealeii* and reflectin-1b from *Euprymna scolopes*. Colored boxes indicate locations of the conserved SD1-6 regions. Underlined areas represent inserted regions found only in Ref-Lp1. Among the reflectins of *E. scolopes*, reflectin-1b (ref-1b) showed highest similarity with the Ref-Lps. Symbols above the amino acid alignment represent residues that are either present in all three proteins (*), in Ref-Lp1 and Ref-Lp2 only (·), or in ref-1b and either Ref-Lp1 or Ref-Lp2 (-).
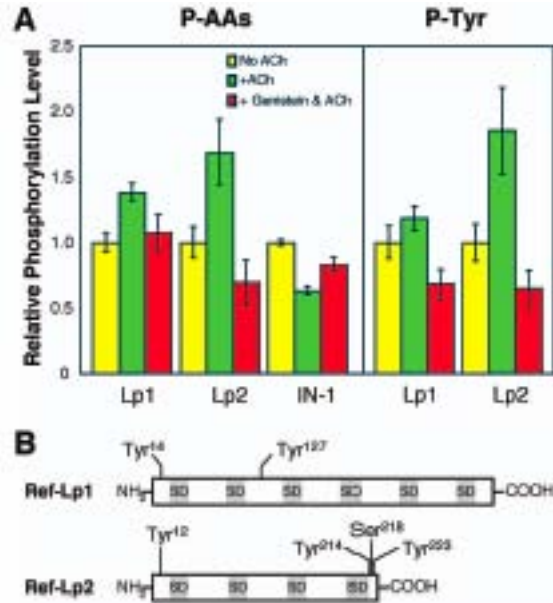
9

Fig. 4. Phosphorylation of Ref-Lp1 and Ref-Lp2 induced by the addition of ACh. (*A*) Phosphorylation was quantified by analysis of 1-dimensional electrophoresis of proteins extracted from samples incubated in the absence (yellow) or presence (green) of Ach; genistein-treated samples also were analyzed (red). Analyses were conducted by staining with Pro-Q Diamond stain for all phosphoamino acids (P-AAs) and Western blotting with PY20, an antibody specific for phosphotyrosine (P-Tyr). The values for ACh-activated and genistein treated samples are normalized to the average values obtained from the inactive reference sample. All values represent averages ± standard deviations based on analyses in triplicate. (*B*) Phosphorylated residues on Ref-Lp1 and Ref-Lp2 were identified by MALDI-TOF MS and their relative positions on each protein are indicated by arrows for the two Ref-Lps.
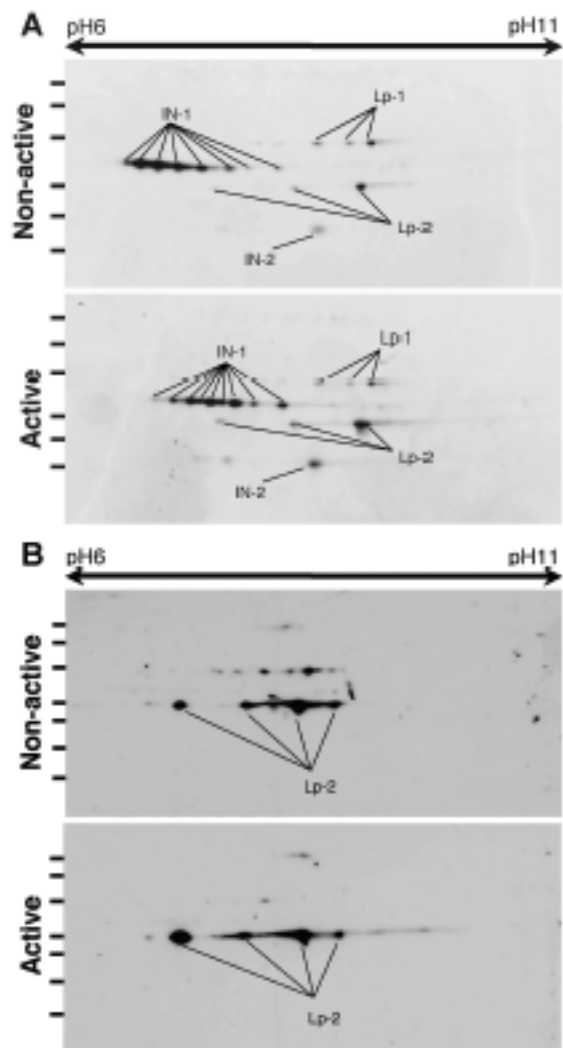
Fig. 5. Phosphorylation of reflectins mapped by 2-D PAGE. Detection of phosphoproteins as conducted by staining with Pro-Q Diamond for all phosphoamino acids (*A*) and by Western blotting with PY20 antibody for phosphotyrosine (*B*); proteins were prepared from non-activated (top) or activated (bottom) samples. (*A*) Pro-Q Diamond staining revealed the presence of many distinct phosphorylated states for Ref-Lp1, Ref-Lp2, IN-1 and IN-2. In the activated state, Ref-Lp2 exhibited higher signal intensity (more phosphorylation), while IN-1 was dephosphorylated. (*B*) Western blotting with PY20 showed that the most acidic species of Ref-Lp2 exhibited higher signal intensities in the activated state. IN-1 and IN-2 were not detected by PY20. Bars indicate molecular weight markers of 64, 49, 37, 26, 19, 15 and 6kDa.
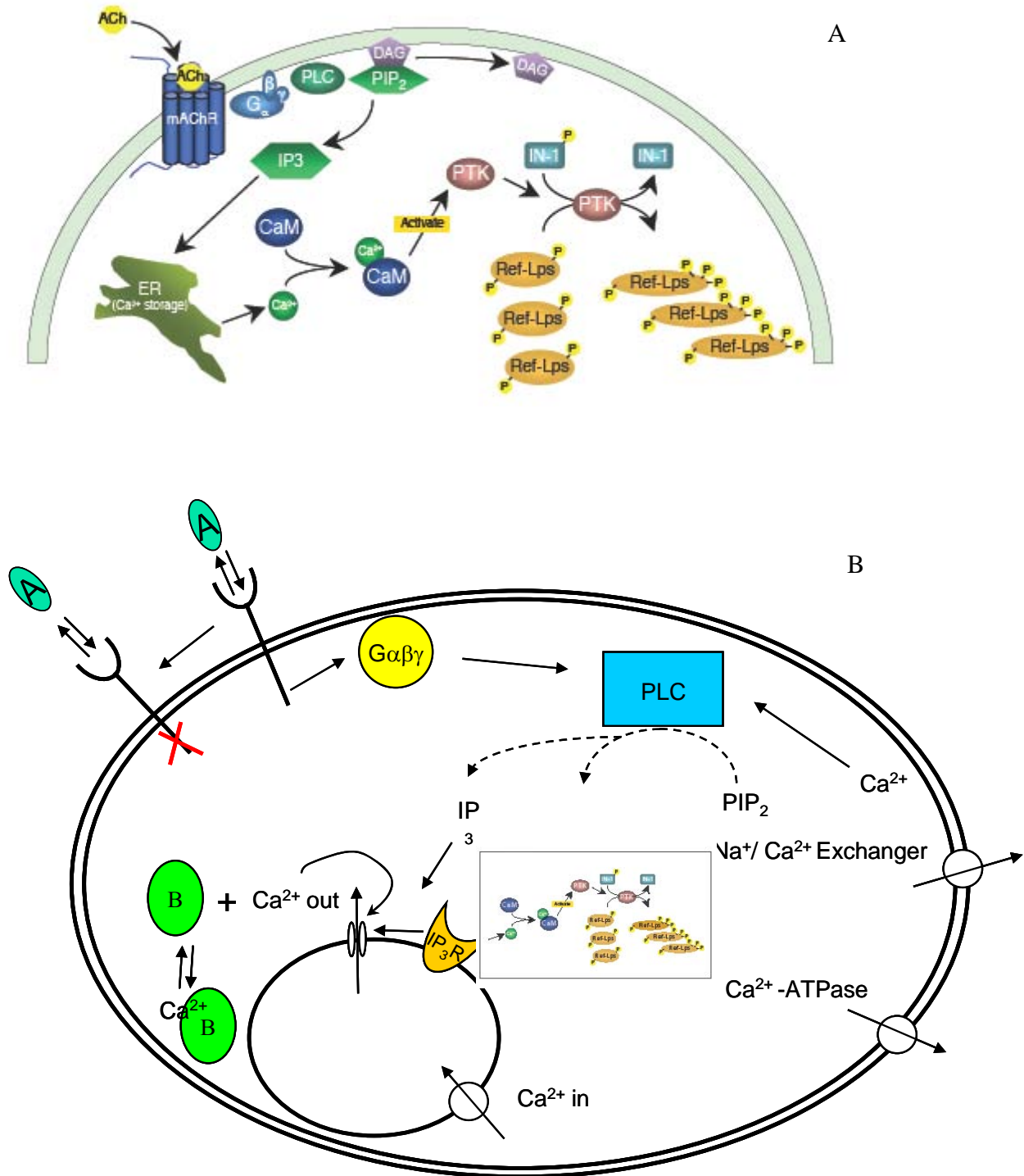
Figure 6. **Acetylcholine activated pathway of reflectin phosphorylation leading to activation of reflectance.** From experimental observations summarized above, we elucidated the pathway of signal transduction culminating in the phosphorylation of reflectin and consequent activation of reflectance (A). A mathematical model of calcium elevation was created by Prof. Doyle and his students that is consistent with this pathway (B).
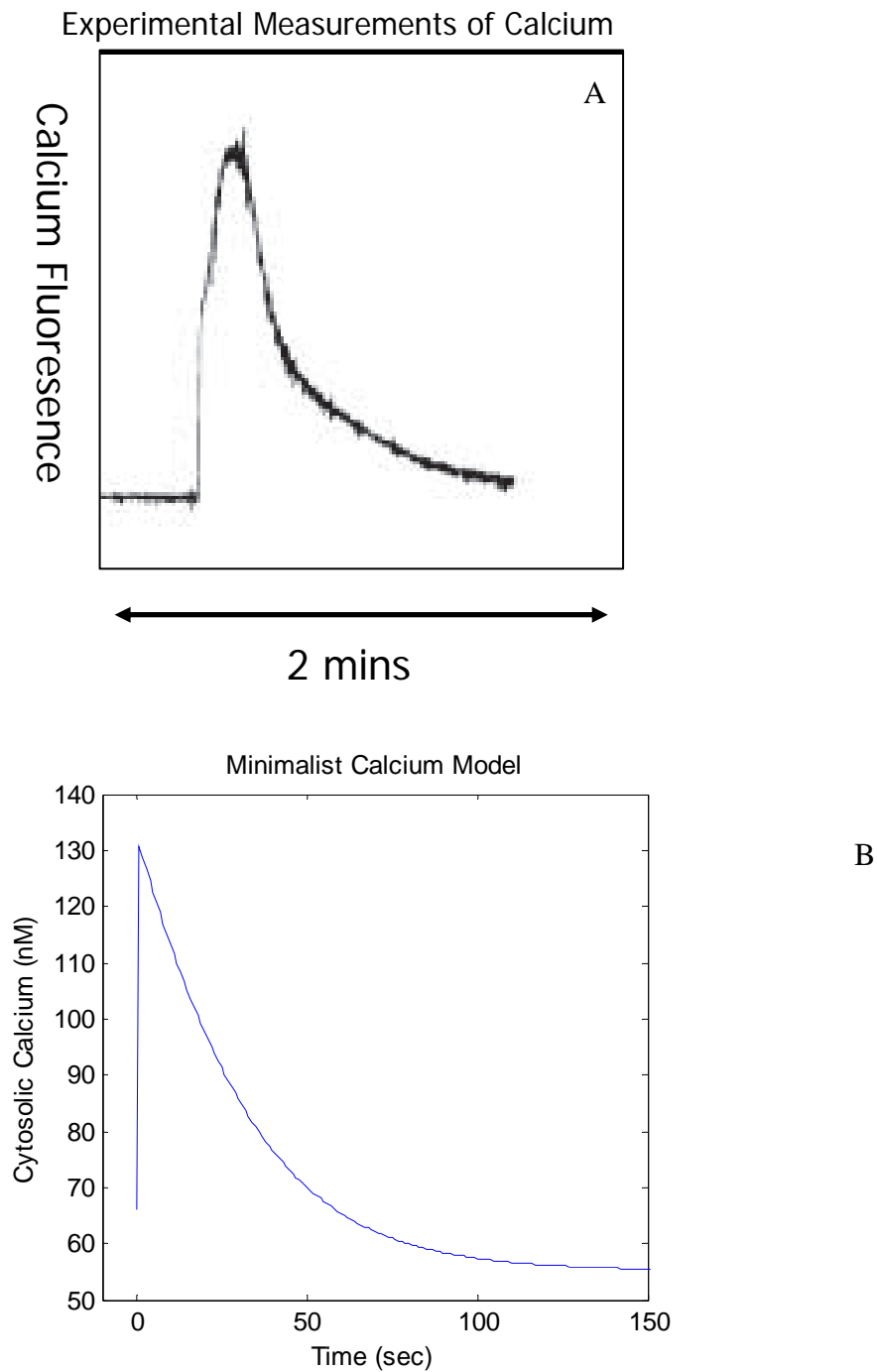
Experimental Measurements of Calcium

A

Calcium Fluoresence

2 mins

Minimalist Calcium Model

B

Cytosolic Calcium (nM)

Time (sec)

Figure 7 Comparison of Model to Experiment. Rise of calcium in response to acetylcholine in experiment (A) and preliminary mathematical model developed in collaboration with Prof. Frank Doyle (B).